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# THE PROTEIN-SPARING ACTION OF UTILIZABLE CARBOHYDRATES IN CULTURES OF CERTAIN SUGAR-FERMENTING ORGANISMS *

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Attention often has been called to the effect of the presence of various sugars on the protein metabolism of bacteria. It is a well-known fact that in cultures of certain organisms in the ordinary sugar-protein media, indol is not formed, gelatin is not liquefied, casein is not digested, and ammonia-production is greatly lessened.

Inasmuch as the decomposition of a carbohydrate is accompanied by the formation of more or less acid, and because the protein changes mentioned are commonly regarded as due to the action of one or more trypsin-like enzymes—that is, enzymes which do not act in acid media—some investigators have inclined to the belief that it is the acid resulting from the decomposition of the sugar, not the mere presence of the sugar itself, which alters the normal progress of the protein changes.

## HISTORICAL REVIEW

Wherry[1] has stated that the proteolytic ferments of bacteria are active only in a medium alkaline to litmus, that it takes but a small amount of acid to hinder their action, which is in accord with the behavior of trypsin, and that when carbohydrates which can be so fermented as to form acids, are present in gelatin, the liquefaction of the latter is inhibited. Glenn,[2] working with different strains of B. proteus and of B. cloacae, came to the same conclusion. Kuhn[3] concluded from his investigations that acids inhibit the liquefaction of gelatin, and Lehmann,[4] in whose laboratory Kuhn's work was done, stated that the inhibition of the liquefaction of gelatin is due to the trypsin-like nature—sensitiveness to acid—of this enzyme. Auerbach,[5] however, prevented the accumulation of the hydrogen ion by the use of magnesium oxid, and thus showed that Lehmann's explanation was no longer adequate. Jordan[6] noted that "the production of a gelatin-liquefying enzyme is not very dependent upon the reaction of the culture medium. It is probable that the reaction is of importance only in so far as it influences the general conditions of growth of the micro-organism."

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[1] Jour. Infect. Dis., 1905, 2, p. 317.
[2] Centralbl. f. Bacteriol., I, O., 1913, 58, p. 481.
[3] Arch. f. Hyg., 1891, 13, p. 70.
[4] Grundriss und Atlas der Bacteriol., 1890, p. 57.
[5] Arch. f. Hyg., 1897, 31, p. 311.
[6] Biological Studies by the Pupils of William Thompson Sedgwick, 1906.

Hirschler,[7] before Auerbach, had shown that phenol, skatol, cresol, indol, and the oxy-acids are not formed in mixed cultures when starch, cane sugar, dextrin, glycerin, or calcium lactate are present, even tho the acid reaction is kept down, as it was in his experiments, by calcium carbonate. He expressed himself on this point as follows: "It is quite probable that the acid resulting from the decomposition of the carbohydrates is the cause of the absence of protein decomposition, but up to the present, we lack proof that this is alone responsible for the hindering effect of the carbohydrates. It is quite probable that the presence of substances which are more easily decomposed than are proteins, is the cause of the absence of protein decomposition. Moreover, it is possible that these easily decomposed substances, as sugar, glycerin, etc., call forth a vigorous development of those varieties (of mixed culture) which decompose these substances, and this in turn influences unfavorably the other varieties which can decompose only the proteins."

Kendall, Day, and Walker,[8] in observations extending over a number of years, have demonstrated the protein-sparing action of utilizable carbohydrates in bacterial metabolism. They regard this protein-sparing action as due, not to the inhibiting action of the acid resulting from the decomposition of the carbohydrate, but to the fact that utilizable sugars serve the fuel requirements of bacteria better than do proteins. Since the fuel requirements of bacteria are so much in excess of their structural requirements, it is evident that a utilizable sugar, by lending itself more readily to oxidation and energy-giving reactions, simply diverts the attack of the bacteria from the protein constituents of the medium, so that the products of decomposition on the protein side of their diet are almost entirely wanting, a slight ammonia-production being the only one in evidence and that merely representing changes in whatever part of the protein is necessary for the structural purposes of the bacteria.

In consideration of this lack of agreement in opinions as to how the presence of the utilizable sugar affects the protein metabolism of the bacteria, the following series of experiments was undertaken to determine (1) in what respects the proteolytic enzymes of bacteria are tryptic in nature, (2) the nature of the conditions which prevent the formation of the enzymes in sugar-protein media, and (3) the nature of the conditions which prevent the action of the enzyme after it has appeared in the cultures.

The conclusions presented are based on observations made on media containing, unless otherwise stated, 1% peptone and 0.5% $NH_4Cl$. Bacillus proteus, as a type of the proteolytic sugar-fermenting group of organisms, was used for inoculations. A sufficient number of other organisms of this type—B. mesentericus, B. prodigiosus, the spirillum of Asiatic cholera—and other combinations of nutrient media were also used so that the conclusions reached might not be open to objection as applying only to B. proteus in media of relatively simple composition.

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[7] Ztschr. f. physiol. Chem., 1886, 10, p. 306.

[8] Jour. Am. Chem. Soc., 1914, 36, p. 1966.

The various reactions of the media used in this work are expressed in terms of the normality system. In view of the fact that the percentage of dissociation of a given normality of a given acid in a medium of given composition is so widely different from that of the same normality of the same acid in water solution, or in a medium of different composition, the distinction between titratable acid and "hydrogen-ion concentration" must not be overlooked, for under ordinary cultural conditions it is the dissociated hydrogen, not the titratable hydrogen, which modifies the progress of changes produced in the media by bacterial enzymes. For example, the maximal amount of titratable acid produced by B. bulgaricus in rennet whey is N/50. The maximal amount produced in skimmed milk is N/6—over 8 times as much as in rennet whey. Yet a measurement of the hydrogen-ion concentration of these two cultures by the electrometric method shows identical degrees of acidity (if active, or dissociated, hydrogen is meant)—a concentration representing a definite and fixed limit for each strain of the organism, and, provided normal growth is obtained, affected neither by the protein content nor by the initial reaction of the medium. The great difference in the amounts of titratable acid in the two media in this case is due to a difference between the so-called buffer content of the whey and that of the skimmed milk. The excess of protein in the skimmed milk simply represses the dissociation of the hydrogen ion of the acids of the carbohydrate-fermentation, thereby protecting the bacteria over a longer period of their vegetative activity, and permitting a greater accumulation of these acids in the molecular, or titratable, form.

A measurement of the titratable acid is, however, an indirect measurement of the dissociated hydrogen, provided the measurement is made each time for a given acid in a medium of approximately constant buffer content. No attempt has been made, therefore, to draw conclusions regarding the concentration of hydrogen ion in one medium, by measuring a like amount of titratable acid in another medium, except in one especially noted case, in which the buffer values of the two media were sufficiently close to warrant the conclusions there reached.

## I

## THE METHOD OF PREPARATION OF THE PROTEOLYTIC ENZYME OF B. PROTEUS

One liter of a 7-day-old $NH_4Cl$-peptone culture of B. proteus was passed through a Berkefeld filter to remove the bacterial bodies. The water of the filtrate was then evaporated off at room temperature over sulfuric acid in a sterile desiccator. The dry residue was rubbed up with 98% alcohol. A large part of the peptone collected as a gummy mass, which was then removed with the stirring rod. Small flocculent particles floating in the alcohol were separated from the $NH_4Cl$ (which is soluble in 98% alcohol) by filtering through filter paper. The alcoholic filtrate when evaporated left a residue (mainly consisting of $NH_4Cl$) which was almost entirely without effect on gelatin. The result showed also that the enzyme is practically insoluble in alcohol. Thus, one after another, the bacteria, the water, a large part of the peptone, and the $NH_4Cl$ were removed from the flocculent material on the filter paper. This substance when removed and dried gave a fine white powder, which liquefied carbol-gelatin in concentrations of 1:10,000. Its activity remained apparently undiminished after months of exposure to the action of light and air. In this respect it differs from trypsin.

## THE DIGESTION OF OTHER PROTEINS WITH THE PROTEOLYTIC ENZYME OF B. PROTEUS

*Casein.*—Five milligrams of this powdered enzyme were added to 5 c.c. of a solution containing 0.5% phenol, 0.1% powdered casein, and 0.1% $Na_2CO_3$. A 5-c.c. tube of casein solution without enzyme was used as a control. After 6 hours at 37 C. the contents of the two tubes were mixed with a few drops of acetic acid (1 part glacial acetic to 100 parts water). Casein was precipitated from the control solution, but not from the solution to which the powdered enzyme had been added, a result which shows that the enzyme can digest casein, at least to the stage where the products of its digestion are not precipitated in acid solution.

In order to test the action of this enzyme on casein in acid solution, small quantities of the powdered enzyme and the powdered casein were shaken in 5 c.c. of N/50 lactic acid. A control tube without the enzyme was also prepared. The two tubes were then placed in the incubator, and shaken at frequent intervals. In about 24 hours the casein enzyme tube became clear, while the casein in the control tube showed no evidence of digestion. In this respect also, this enzyme is unlike trypsin, which can digest casein only in approximately neutral or alkaline solutions.

*Fibrin.*—Finely divided fragments of fibrin were shaken up with some of the powdered enzyme in N/50 lactic-acid and N/50 sodium-hydroxid solutions. These, together with controls, were incubated at 37 C., with frequent shaking. In the tube containing the enzyme in alkaline solution digestion of the fibrin particles was complete in 8 hours; in that containing the acid solution, in 24 hours. The control tubes showed no digestion.

*Blood Corpuscles.*—A 2% suspension of washed blood corpuscles of the dog was divided into two 5-c.c. portions. Some of the powdered enzyme was inactivated by heating in the autoclave at 120 C. for 30 minutes, and then mixed with one of the suspensions for a control. The other suspension was mixed with an equal amount of the unheated enzyme, and the two tubes kept at 37 C. After 2 hours hemolysis was complete in the suspension containing the unheated enzyme, while the control suspension showed a clear supernatant fluid. Subsequent examination of the hemolyzed suspension with the microscope showed that the stroma of the cells had been digested. It appears from these experiments that the proteolytic enzyme of B. proteus, elaborated in the relatively simple medium of $NH_4Cl$ peptone, has a digestive action on substances representing a rather wide range of protein characteristics, and for this reason should be considered a true protease rather than a "glease," as it has often been termed.

### THE EFFECT OF HEAT ON THE STABILITY OF THE PROTEOLYTIC ENZYME OF B. PROTEUS

About 50 c.c. of a 48-hour $NH_4Cl$-peptone culture were boiled in a beaker over a flame, 1-c.c. portions being removed at intervals of 1, 5, 10, and 30 minutes, and mixed with 5 c.c. of carbol-gelatin (0.5% phenol in 15% gelatin). Other portions of the culture were heated in the autoclave for periods of 5 and 15 minutes at 120 C., and 1 c.c. of each portion was mixed with 5 c.c. carbol-gelatin. For controls, a tube containing 5 c.c. of gelatin with 1 c.c. of unheated culture and another with 1 c.c. of water were prepared. After 24 hours' incubation at 37 C. the tubes were removed from the incubator and allowed to cool to room temperature. The tube to which only water had been added, and the tube containing the portion of enzyme which had been heated in the autoclave at 120 C. for 15 minutes solidified at about the same time; next, the tube containing the enzyme which had been heated for 5 minutes at 120 C.; then the tube containing the enzyme of the 30 minutes' boiling; and last, after about 6 hours, the tube containing the enzyme of the 10-minutes' boiling. None of the other tubes became solid, even when placed in the ice-box, a fact showing that boiling for 5 minutes has no noticeable effect on the gelatin-digesting power of this enzyme. In this respect, again, it is unlike trypsin, which is destroyed before the boiling point is reached.

The method of heating was used also in testing the stability of the casein- and fibrin-digesting property of the enzyme of B. proteus. It was found that only after heating at 120 C. for 15 minutes was complete destruction obtained.

In using this method of determining the activity of the enzyme on gelatin, it is necessary that the tubes containing the gelatin be of the same diameter and thickness of glass, and in some cases it is even well to check up their order of solidifying by exchanging their contents.

### THE DIFFUSIBILITY OF THE PROTEOLYTIC ENZYME OF B. PROTEUS

Two test tubes were suspended with their closed ends downward, about 1 cm. apart, within a beaker, the closed ends reaching to within 1 cm. of the bottom. Melted carbol-agar was then poured around them and allowed to solidify. The tubes were then carefully loosened and withdrawn from the agar, leaving 2 holes about 5 cm. deep. Carbol salt solution was poured into each hole and a small quantity of the powdered enzyme stirred into one of the holes. The salt solution in the other hole, after 24 hours, was able to digest gelatin, casein, and fibrin, proving thereby that the proteolytic enzyme of B. proteus still retains its activity on various proteins after diffusion through 1 cm. of solidified (2%) agar.

### IS THE PROTEOLYTIC ENZYME OF B. PROTEUS A UNIT OR A MIXTURE OF ENZYMES?

From the foregoing experiments and others to be described later, it appears that the action of heat on the enzyme affects the ability of the enzyme to digest gelatin, casein, and fibrin to about the same degree in each case. Similarly, the dialyzed product can act on all three of these proteins. Strongly acid reactions (N/20 lactic) retard, and alkaline reactions (N/50 to N/20 NaOH) accelerate the digestion of all three. Casein, fibrin, and gelatin are digested more quickly with a given quantity of $NH_4Cl$-peptone culture than with NaCl-peptone culture, while none of them is digested by $NH_4Cl$-peptone cultures containing 0.7% of calcium lactate. In other words, acids, alkalies, $NH_4Cl$, calcium lactate, heating, and dialyzing seem to affect the ability of the enzyme to digest these various proteins to the same degree. Such facts indicate that the enzyme is probably a unit, not a mixture of enzymes. The unity of trypsin is disputed by Pollak[9] because of its ability to digest gelatin in media of slightly acid reaction. Thus far it is seen that the only essential property which the enzyme of B. proteus has in common with trypsin is its ability to digest proteins of widely different natures. Other greater differences are presented in the experiments that follow.

## II

### THE AMOUNT OF ACID PRODUCED BY B. PROTEUS IN $NH_4CL$-PEPTONE MEDIA CONTAINING DIFFERENT AMOUNTS OF GLUCOSE

One-hundred-cubic-centimeter flasks of $NH_4Cl$-peptone media containing 0, 0.1, 0.2, 0.3, 0.3, 0.4, and 1% of glucose were inoculated with B. proteus and placed in the incubator; 10-c.c. portions were removed every 24 hours and titrated with N/10 NaOH to determine the rate of increase of acid. Also, 1-c.c. portions were removed every 12 hours and mixed with 5 c.c. carbol-gelatin to determine the time of the appearance of the proteolytic enzyme.

*Results.*—(a) The flask containing no sugar showed no appreciable change in reaction after the first 24 hours. The enzyme was present at that time .

(b) The flask containing 0.1% glucose showed an acid reaction of approximately N/180 acid to litmus after 24 hours, and also a slight digestive action on gelatin.

(c) The 0.2% glucose flask showed its maximal acidity of approximately N/100 after 28 hours. The enzyme was not present until the 3rd day.

(d) The 0.3% glucose flask showed a reaction of N/70 after 72 hours, but the enzyme did not appear until the 5th day.

(e) The 0.4, 0.5, and 1% glucose flasks showed approximately equal amounts of acid (N/50) after 3 days, but no enzyme appeared in these flasks, even after 14 days.

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[9] Hofmeister's Beiträge, 1904-5, 6, p. 95.

From the results obtained with the 0.1, 0.2, and 0.3% glucose flasks, it is seen that the proteolytic enzyme can be elaborated in media of a decidedly acid reaction, and that the order of appearance of the enzymes in these flasks is in the reverse order of the amounts of sugar present; that is, the enzyme appears first in the flasks containing the smaller amounts of glucose. From the results obtained with the 0.4, 0.5, and 1% glucose flasks, it appears that even tho an excess of sugar is present, B. proteus does not continue its action on carbohydrates in acid reactions above approximately N/50 acid to litmus. The reason for this will be shown in a subsequent experiment.

## THE TIME ELAPSING BETWEEN THE DISAPPEARANCE OF A UTILIZABLE SUGAR FROM THE CULTURE AND THE APPEARANCE OF THE PROTEOLYTIC ENZYME

Flasks containing 200 c.c. of $NH_4Cl$-peptone medium to which 0.2% glucose had been added were inoculated with B. proteus and placed in the incubator. The rate of disappearance of the sugar was followed by removing 10-c.c. portions of the culture at regular intervals, and titrating by Bang's method of sugar-estimation. The sample of peptone used in this experiment gave a positive Molisch reaction even after 2 weeks' incubation with B. proteus. Altho this protein has a reducing action on the copper solution, the amount of reduction it causes was assumed to occur as a constant factor in the titration of each sample, since it apparently does not diminish in quantity in cultures of B. proteus.

TABLE 1

An Average of the Records of Observation on Cultures of B. Proteus

| Sample | Hours After Incubation | Amount in c.c. of Hydroxylamin Solution | Percentage of Dextrose* | Liquefaction of Gelatin | Amount in c.c. of N/10 NaOH Required to Make 10 c.c. of Culture Neutral to Litmus |
|---|---|---|---|---|---|
| 1 | Initial titration | 29.0 | .196 | — | 0.0 |
| 2 | 8 | 30.0 | .186 | — | 0.2 |
| 3 | 24 | 36.5 | .119 | — | 0.6 |
| 4 | 32 | 46.5 | .03 | — | 1.1 |
| 5 | 48 | 50.0 | 0 | + | 1.0 |

* Hammarsten: Text-Book on Physiological Chemistry, 1911, p. 765.

The strain of B. proteus used in these experiments was able to remove 0.2% glucose from these cultures in from 32 to 40 hours. By removing 1-c.c. portions at regular intervals and mixing with 5 c.c. carbol-gelatin it was found that the enzyme in no case appeared before the sugar was entirely removed from the culture, and usually appeared from 10 to 12 hours afterward.

It was thought that the appearance of the enzyme could be further delayed by using a larger percentage of sugar, and adding $CaCO_3$ to the media to neutralize the acid as formed. Accordingly, by following the rate of the disappearance of the sugar in cultures containing 0.5% dextrose and 0.5% $CaCO_3$ it was found that the entire quantity of sugar had disappeared on the 4th day after incubation. The appearance of the enzyme in this culture was tested by mixing 1-c.c. quantities of it with 5 c.c. carbol-gelatin, as in the case of the 0.2% dextrose culture already described. This was done daily for 14 days, but no formation of the enzyme was detected. The failure of the enzyme to appear in the $CaCO_3$ media after the sugar had disappeared, recalled the statement made by Hirschler[10] that calcium lactate prevented the formation of indol, phenol, etc., a fact which he interpreted to mean that this substance is used for fuel purposes (the lactate ion being reduced to the propronate ion) and therefore has an action similar to that of the carbohydrates.

Inasmuch as calcium lactate is formed in such a culture as described —by the interaction of lactic acid and $CaCO_3$—the experiment of determining whether or not the presence of calcium lactate affects the metabolism of B. proteus in sugar-free and sugar-containing media suggested itself.

## EFFECT OF LACTATE SALTS ON THE METABOLISM OF B. PROTEUS

(a) Fermentation tubes were filled with a medium containing 0.5% $NH_4Cl$, 0.5% glucose, and 1% peptone. To these tubes were added 0.1%, 0.2%, and so on up to 1% calcium lactate, and the series inoculated with B. proteus. After a 24-hour incubation, gas- and acid-production were almost completely inhibited in media containing as much as 0.7% of calcium lactate. The experiment was repeated with the ammonium and sodium salts of lactic acid. These were also found to have an inhibiting effect on the gas- and acid-formation.

The absence of any appreciable change in the reaction of these cultures is evidence that the lactic acid salts not only do not serve the fuel requirements of B. proteus, but also prevent in some way its normal utilization of the sugars. Enzymic actions in general are retarded, or even suspended entirely, by the presence of the products of reactions caused by a given enzyme, and this fact may serve to explain why the presence of these lactate ions inhibits the action of the endo-enzymes of B. proteus.

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[10] Ztschr. f. physiol. Chem., 1886, 10, p. 306.

It will be shown in a subsequent experiment that the other ion of the lactic-acid molecule, namely, the hydrogen ion, has a similar effect —retarding and finally completely suspending the metabolic activities of endo-enzymes of B. proteus.

(b) Five tubes containing 10 c.c. $NH_4Cl$-peptone medium, and 5 tubes of the same medium to which 0.7% calcium lactate had been added, were inoculated with B. proteus and incubated. After 5 days the ammonia-production, as determined by the Folin air-current method, was approximately 5 times as great in the cultures which did not contain calcium lactate as in those which did, the result showing that this salt in some way interferes with the utilization of protein for fuel purposes. Furthermore, it also prevents the formation of the proteolytic enzyme, even after a 14-day incubation—the same observation which was made in the experiment described on p. 40. Indol was also absent in the cultures containing lactic-acid salts, as was observed by Hirschler in 1886. Macroscopic observation of the cultures, however, revealed no appreciable difference in the amount of growth in the two sets of tubes.

### WHY IS THE PROTEOLYTIC ENZYME OF B. PROTEUS NOT FORMED IN CULTURES CONTAINING GLUCOSE?

The work of Kendall, Day, and Walker[11] gives two reasons why bacteria show a preference for utilizable sugars in ordinary sugar-protein media. In the first place, the protein molecule requires a digestion to simpler fragments before diffusion into the interior of the cell can occur, and since the oxidation of energy-giving reactions is brought about by the endo-enzymes of bacteria, these protein molecules can not be used for fuel as long as they can not diffuse into the interior of the cell. Sugar, on the other hand, readily diffuses into the cell body where it can be acted on by the zymase or endo-enzyme. In the second place, even tho protein in the digested form is added to sugar-protein media, its fragments, such as the amino-acids, etc., are more difficult to oxidize than are the utilizable sugars. In other words, the difference in the ability of these two foods, protein and sugar, to pass through the cell wall of the bacteria, and the difference in the readiness with which they are oxidized, are the two reasons why bacteria show a preference for sugar in protein-sugar media.

Unless an excess of sugar is present, this preference for sugar first causes the culture to become progressively acid in reaction as long as the sugar lasts. When the supply of the latter is exhausted, the bacteria are forced to change from a carbohydrate to a protein diet for fuel purposes. The products of protein-decomposition, such as indol, skatol, etc., then appear in the culture. Ammonia, being one of the decomposition products resulting from a utilization of the proteins, causes a gradual decrease in the acid reaction until the culture may eventually become alkaline.

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[11] Jour. Med. Research, 1911, 25, p. 155.

On the other hand, if more sugar is present than the bacteria can remove, the culture eventually reaches a condition, the nature of which is demonstrated in the following experiment.

A flask of 100 c.c. of $NH_4Cl$-peptone medium containing 1% glucose was inoculated with B. proteus and incubated at 37 C. until fermentation ceased, or for about 3 days. A portion of the culture was then filtered through a Berkefeld filter, and the bacteria-free filtrate removed with a sterile pipet to 2 sterile fermentation tubes. These two tubes were inoculated with a portion of the original 3-day-old culture. The bacteria used for the inoculation were then back in the medium from which they had been filtered—a medium which apparently contained something inhibiting the action of their sugar-fermenting enzyme. One of the tubes was neutralized with sterile N/10 NaOH, and the two tubes placed in the incubator. After 24 hours the tube which had been neutralized, showed the usual amount of growth, of gas, and of acid-formation, while the medium in the other tube, even after 2 weeks' incubation, remained perfectly clear and showed no gas-formation.

Since both growth and sugar-decomposition—that is, anabolism and catabolism—had occurred in the tube which had been neutralized, and were both absent in the other tube, it is evident that the bacteria had produced something which had suspended both functions of their metabolism—the building up and the tearing down of organic substances. Titratable acid in $NH_4Cl$-peptone media in concentration of N/50 acid to litmus seems to paralyze completely the action of the endo-enzyme of these bacteria, so that, altho an excess of energy-giving substances is present, the bacteria cannot appropriate them. In other words, when their endo-enzymes become paralyzed by certain products of their own reaction, the bacteria are virtually starved into a dormant condition.

## III

### THE LIMITS OF ALKALINITY AND ACIDITY IN WHICH THE PROTEOLYTIC ENZYME OF B. PROTEUS CAN LIQUEFY GELATIN

In the previous experiments it was shown that titratable acid in broth in concentration of N/50 acid to litmus practically suspends the vegetative and reproductive functions of B. proteus. Since the bacteria cannot even multiply in this concentration of acid, the conclusion is warranted that this acidity (N/50) is the limit of the acid reaction in broth in which the organisms can elaborate their protein-digesting enzyme. It does not necessarily follow, however, that this is also the limit of acidity which would still permit the action of this enzyme after it had been formed. In other words, the limit of acidity of the culture which still permits the formation of the enzyme, and that which still permits the activity of the enzyme after it is formed, are not necessarily the same, because the proteolytic enzyme is an extracellular product, and is not so closely associated with the life of the cell itself, as is the intracellular, sugar-decomposing enzyme. The following experiment, therefore, was undertaken to determine the stability of the enzyme with reference to acids and alkalies.

Carbol-gelatin of N/20, N/30, and N/40 titratable alkali (NaOH), and of N/20, N/30, and N/40 titratable acid (lactic) was prepared, and 5 c.c. of each of these placed in each of two tubes. With the content of one tube of each pair was mixed 1 c.c. of a 48-hour $NH_4Cl$-peptone culture of B. proteus, and with that of the other one of each pair, 1 c.c. of water, for controls. After 18 hours at 37 C. the tubes were removed from the incubator and allowed to cool to room temperature. The controls without the enzyme, all solidified in approximately the same length of time (15 minutes), a result showing that no hydrolysis of the gelatin, due to the action of the acid or alkali, had taken place. Of those tubes containing the 1 c.c. of culture—that is, of the enzyme—none showed any tendency to become solid after standing 2 hours, a result showing that even in dilutions of 1 : 6, this enzyme is able to digest gelatin to the point of liquefaction in 18 hours, in media to which has been added titratable acid in concentrations of N/20 acid to litmus.

In previous experiments, it was demonstrated that B. proteus can not form this enzyme in 1% peptone in hydrogen-ion concentration greater than that corresponding to N/50 titratable acid. The immediately foregoing experiment, however, shows that much greater acid reactions than N/50 do not prevent the action of this enzyme after it is once formed. This may be considered as further evidence that the failure of B. proteus to liquefy sugar-gelatin cultures is not due to the acid reaction of the culture, because this acid reaction reaches a maximum of only approximately N/50, whereas B. proteus is known to be active in acid reaction of as much as N/20, and that, too, in dilution of 1 part of culture to 5 parts of gelatin.

It has been stated before that the active agent affecting the progress of changes produced by bacteria and their enzymes is the dissociated hydrogen and not the titratable acid. It should be noted here, however, that the 15%-gelatin-0.5%-carbol mixture used in this test, and the glucose-$NH_4Cl$-peptone media used in Experiment II have approximately the same tendency to repress the dissociation of the hydrogen ion of lactic acid. The great difference, therefore, between the amount of titratable acid in the N/20-acid gelatin of this experiment and in the N/50-acid broth of Experiment II(e) is sufficient evidence that a correspondingly great difference in the hydrogen-ion content of the two media also exists. Objection that the enzyme can not be elaborated in an acid reaction, or else is destroyed in some stage of its formation can hardly be made. Both Auerbach[5] and Hirschler[7] kept their cultures neutral with MgO and $CaCO_3$ and still found no protein-decomposition taking place in sugar-containing media. Furthermore, the 0.1, 0.2, and 0.3%-glucose flasks of Experiment II were all of distinctly acid reaction for several hours or days, and in spite of this the organisms were able to form the enzyme after the sugar had been exhausted.

It might be asserted that some by-product of the sugar-decomposition other than the hydrogen ion is responsible for the failure of sugar-gelatin cultures to become liquefied. To determine whether this is the case, the following experiments were performed:

(a) One 10-c.c. tube of sugar-free nutrient gelatin and 2 tubes of 1% glucose gelatin were inoculated with B. proteus. After 24 hours the sugar-free gelatin was liquefied. The two 1%-glucose-gelatin tubes were still solid, and remained so, even after 14 days. When the three tubes had been incubated for 14 days, 1 c.c. of the liquefied gelatin of the glucose-free culture (containing the enzyme), was transferred to one of the 1%-glucose-gelatin cultures and both glucose-gelatin cultures returned to the incubator. After 6 hours at 37 C. the sugar-gelatin tube to which the 1 c.c. of sugar-free gelatin had been added was liquefied, in spite of its acid reaction, while the other glucose-gelatin tube was still solid.

(b) This experiment was repeated with two 10-c.c. tubes of milk to which 1% of glucose was added, and one plain milk tube. After a 7-day incubation with B. proteus 1 c.c. of the plain culture (which was almost completely peptonized) was added to one of the 1% glucose-milk tubes. The two glucose-milk cultures had been coagulated by the acids formed from the glucose, but they showed no signs of peptonization. After 3 days in the incubator the glucose-milk tube, to which the 1 c.c. of plain-milk culture had been added, was well peptonized, while the other showed no such change. The undigested curd from the first tube weighed only 0.08 gm., while that from the other tube weighed 0.45 gm.

These experiments apparently justify the same conclusion; namely, that the absence of protein changes in sugar-containing media is not to be attributed to the inhibiting effect of hydrogen ion, or some other substance, or to the action of a trypsin-like ferment, but simply to the fact that the enzyme which makes possible the utilization of the proteins for fuel purposes does not appear in the culture as long as a utilizable carbohydrate is present.

## SUMMARY

The proteolytic enzyme of Bacillus proteus is insoluble in 98% alcohol. The dried enzyme is active after months of exposure to light and air, and is not completely destroyed by boiling for 30 minutes; it is diffusible through one-half inch of 2% agar; it acts best in alkaline solution, but can digest fibrin gelatin and casein in solutions of N/50 or more acid reaction. Failure to destroy its power to digest one protein while leaving intact its ability to digest another indicates that it is a unit rather than a mixture of enzymes.

B. proteus incubated in sugar-containing medium until it has produced its maximal acidity, refuses to grow when re-inoculated into this medium filtered. This failure to grow is probably to be attributed to the paralyzing effect of hydrogen ion on the endo-enzyme of the bacteria, since subsequent neutralization of the media restores the vegetative and reproductive functions of the bacteria. The vegetative function is also hindered by the presence of the lactate ion, either as sodium, ammonium, or calcium salts, but to a less degree than in the case of the hydrogen ion.

Nutrient gelatin to which has been added as much as 0.5% of a utilizable sugar becomes acid after a few hours' incubation with B. proteus, and the gelatin of the medum is not liquefied, even after weeks or months of incubation. The constant concurrence in cultures of certain bacteria of these two phenomena—the acidity of the cultures on the one hand, and the absence of the proteolytic changes in the gelatin, or other protein, on the other—seems to permit but two possible explanations. Either (1) the gelatin-liquefying enzyme of B. proteus, and of certain other bacteria, is trypsic in nature and therefore unable to act in the presence of the acid of the cultures, or else (2) the enzyme is not present in cultures containing a utilizable carbohydrate. The fact that a small quantity of the enzyme, either as the dried powder or in culture form, when added to a sugar-gelatin culture that has reached its maximal acidity, causes liquefaction of this culture after 6 hours at 37 C., indicates that this enzyme acts readily in cultures of acid reaction, and that the second explanation is therefore the more plausible—namely, that the absence of the proteolytic changes in sugar-containing media is due to the fact that the enzyme causing these changes does not appear in cultures containing a utilizable carbohydrate.